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BACULOVIRAL EXPRESSION OF RECOMBINANT S1 DOMAIN OF THE PORCINE EPIDEMIC DIARRHEA VIRUS SPIKE (PEDV-S1) PROTEIN OF HIGHLY PATHOGENIC PEDV IN INSECT CELLS

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ABSTRACT

Background: Highly pathogenic Porcine epidemic diarrhea virus (PEDV) is causing damages to the swine industry and is responsible for loss of economics. Vaccination is the most effective method to prevent and control PEDV infections.
Methods: Recombinant virus-like particles (VLPs) by baculovirus expression vector system have been suggested as a promising platform for new viral vaccines.
Results: In this study we constructed a recombinant baculovirus that was potent to express PEDV-S1 protein; PEDV-S1 protein (S1) which is essential to generate immunogenic VLPs in insect cells. A triplet cassette providing simultaneous and independent expression of S1 gene of PED virus was designed and subjected to synthesis. The cassette was then cloned into pFastBacHTb plasmid and then transformed in to competent Escherichia coli DH10Bac cells and the recombinant bacmids were produced following the homologous Tn7 site-specific transposition. This construction was verified by polymerase chain reaction (PCR) and then transfected into Sf9 insect cells to package new recombinant baculovirus expressing complex proteins of the interest. Restriction map of pFastBacHTb_PEDV-S1 plasmid confirmed the fidelity of the clone. The PCR carried out on the recombinant bacmids as template indicated that a proper homologous recombination has occurred between pFastBacHTb_PEDV-S1 donor plasmid and the bacmid. Following the transfection of new recombinant bacmids to Sf9 cells, cytopathic effects were observed which indicating the successful packaging of recombinant baculovirus. Protein analysis of the infected Sf9 cells showed that all target proteins were efficiently expressed at the same time.
Conclusion: The recombinant baculovirus constructed in this work has proper characteristics to produce PED virus-like particles in Sf9 cells

INTRODUCTION

Porcine epidemic diarrhea virus (PEDV) is the causative agent of PED, a highly contagious disease of pigs characterized by acute watery diarrhea, and vomiting. The disease has mortality as high as 100 % in newborn piglets, and infected pre-weaning pigs often succumb to severe dehydration (Jung and Saif, 2015a, b; Song and Park, 2012). Since its first appearance in Europe, PEDV outbreaks have occurred persistently in Asia and recently in North America, resulting in enormous economic loss worldwide to the swine industry (Pasick *et al.,* 2014;Vlasova *et al.,* 2014; Wang *et al.,* 2014). PEDV is an enveloped RNA virus possessing a single-stranded, positive-sense genome with a 5′ cap and a 3′ polyadenylated tail and belonging to Alphacoronavirus genus in the family Coronaviridae (Masters, 2006; Park *et al.,* 2012). Based on the entire genome sequence of the well-characterized CV777 strain, PEDV genome is approximately 28 kb, bearing at least seven overlapping open reading frames (ORF) encoding non-

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structural proteins including replicase1a, 1b and ORF-3, and structural proteins including spike (S), envelope (E), matrix (M) and nucleocapsid (N) proteins (Kocherhans *et al.,* 2001). The S protein of PEDV is a type I membrane glycoprotein composed of 1,383 to 1,386 amino acids (aa), depending on the strain. It contains a putative signal peptide (aa 1-24), a large extracellular region, a single transmembrane domain (aa 1,334-1,356), and a short cytoplasmic tail. Although PEDV has an uncleaved S protein because it lacks a furin cleavage site, the S protein can be divided into S1 (aa 1–735) and S2 (736-the last aa) domains based on homology with S proteins of other coronaviruses (Duarte and Laude, 1994; Jackwood *et al.,* 2001; Lee *et al.,* 2010b; Sturman and Holmes, 1984). Like other coronavirus S proteins, the PEDV S protein is known to play a pivotal role, interacting with the cellular receptor to mediate viral entry and inducing neutralizing antibodies in the natural host (Bosch *et al.,* 2003; Chang *et al.,* 2002). More precisely, previous studies have shown that the S1domain includes the main neutralizing epitopes and the receptor-binding region (Lee *et al.,* 2011; Sun *et al.*, 2007).

Furthermore, along with the full-length S gene, the S1 portion is known to be a suitable region for determining genetic relatedness among the different PEDV isolates and for developing differential diagnostic assays *(Chen et al.,* 2014; Lee *et al.,* 2010a). Considering these molecular and biological features of the S1 domain, it would be an appropriate target for developing effective vaccines against PEDV. For yet unknown reasons, efforts to productively propagate PEDV in mammalian cells have remained largely unsuccessful. Although it was shown that PEDV could be propagated in African green monkey kidney (Vero) cells with treatment of trypsin (Hofmann and Wyler, 1988; Shirato *et al.,* 2011; *Wicht et al.,* 2014*)*, the described method appears inapplicable to most isolates (Oka *et al.,* 2014). Since multiple passages are usually required to obtain high-growth PEDV, only a few cell-adapted strains have been reported *(Kusanagi et al., 1992*; Kweon *et al.,* 1999).

During the past decade, studies have identified porcine aminopeptidase N (pAPN) or CD13 as a receptor of PEDV by showing that expression of pAPN in non-permissive cells enabled them to support productive PEDV infection (Li *et al.,* 2007; Oh *et al.,* 2003). Moreover, high levels of pAPN expression were reported to be a critical prerequisite for PEDV infection as engineered swine testicular (ST) cells expressing enhanced levels of pAPN could substantially support productive PEDV replication compared to the non-permissive wild-type counterpart (Nam and Lee, 2010). In this study, we constructed a new recombinant polycistronic baculovirus which is basically required for influenza VLP production in insect cells by using Bac-to-Bac system. This new recombinant baculovirus, simultaneously and independently expresses the main component of PEDV-S1 protein which is specific for highly pathogenic PEDV.

MATERIALS AND METHODS

Cassette Designing and Cloning Strategy

A cassette consisted of PEDV-S1 sequences of highly pathogenic PEDV (Pingtung, Taiwan), regulatory genetic elements such as promoters and polyadenylation signals, as well as spacer sequences containing appropriate restriction sites regarding the cloning scheme of full "PEDV-S1 cassette" was designed and thoroughly synthesised (Genescript, Taiwan). As demonstrated in Figure 1, from 5' to 3', the first open reading frame (ORF) is S1 (1500 bp) that following the cloning of PEDV-S1 cassette will place downstream of polyhidrin (Chuang et al.) promoter belongs to pFastBacHTb donor plasmid. There is a SV40 polyadenylation sequence after PED which improves the level of expression. Then the NA transcriptional unit has designed which starts with polh promoter sequence followed by the HTb (4856 bp) and SV40 polyadenylation sequences. Behind this unite there is the next sequence of polh promoter and S1 in its downstream. After the cloning of cassette into pFastBacHTb plasmid, the plasmid SV40 polyadenylation signal will position in downstream of S1 (1500bp) and constitute the S1 transcriptional unit. The pFastBacHTb_PEDV-S1 cassette was cut by EcoRI/SphI double digestion and cloned into pFastBacHTb through the same restriction sites to construct "pFastBacHTb_PEDV-S1 "donor plasmid.

Generation of Recombinant Bacmid

The recombinant pFastBacHTb_PEDV-S1 donor plasmid was transformed into the Escherichia coli DH10Bac competent cells for Tn7 site-specific transposition of the PEDV-S1 cassette fragment to a bacmid DNA through lacZ gene disruption. The transformed cells were plated onto the LB agar containing Kanamycin 50 µg/mL, Gentamicin 7 µg/mL, Tetracycline 10 µg/mL, Bluo-gal 100 µg/mL and Isopropylthio-β-Galactoside (IPTG) 40 µg/mL and incubated at 37ºC for 48 hours. The high-molecular-weight bacmid DNA was isolated from the overnight cultures by alkaline lysis purification according to the instructions supplied by the manufacturer (Invitrogen, USA). Successful transposition was verified by PCR analysis using either M13/pUC or specific primers for S1 region with the following sequences: M13/pUC forward, 5'-CCCAGTCACGACGTTGTAAAACG-3', M13/pUC reverse, 5'-AGCGGATAACAATTTCACACAGG-3' and S1 forward, 5'-ATGAGTCTTCTA ACCGAGGTC-3'. Amplification reactions were performed under the following condition: 3 minutes at 95ºC followed by 35 cycles of 1 minute at 95ºC for denaturation, 1 minute at 58ºC for primer annealing and 4 minutes at 68ºC for extension, concluded by a final extension step at 68ºC for 10 minutes. Dream Taq™ DNA Polymerase (Fermantas, Lithuania) optimized for robust amplifications on long targets was used in PCR mixture.

Cell Culture and Transfection to Generate Recombinant Baculovirus

Spodoptra Frugipdera (Sf9) cells as adherent culture was purchased from the Cell Bank (Pasture Institute of Iran) and grown in Grace's medium supplemented with 10% fetal bovine serum (Gibco, USA). Sf9 cells were transfected with the isolated recombinant DNA using Cellfectin (Invitrogen, USA) for the generation of the recombinant baculovirus according to the manufacturer instruction. The transfected cells were incubated at 27ºC for 72 - 96 hours, to allow baculovirus assembly and release into the culture media. Recombinant baculovirus production was monitored daily by

visualization of the cytopathic effects. The culture medium was collected, clarified by low-speed centrifugation for 10 minutes at 1000 rpm and subjected to multiple rounds of virus propagation.

Expression and Determination of Recombinant Protein

The overnight cultured Sf9 cells were inoculated with recombinant baculovirus at a multiplicity of infection (MOI) of 10 and incubated at 27ºC for 72 hours. The culture medium (supernatant) and the cells (pellet) were collected after centrifugation. To prepare cell lysate, the pelleted cells were washed three times with cold PBS, suspended in sample buffer (50 mMTris-HCl, pH 8.5, 5 mM 2-mercaptoethanol, 100 mM KCl, 1 mM PMSF) and sonicated three times for 10 seconds with 3 minute-intervals, at 50% power using ultrasonicator (UP 400 s). After centrifugation at 12000 rpm for 15 minutes at 4ºC, the clarified supernatant was stored at -20ºC for further analysis. Uninfected Sf9 cells which were similarly treated were used as a negative control. The protein samples were examined by SDS-PAGE and Coomassie staining (15).

The separated proteins on SDSPAGE were also transferred to nitrocellulose membranes using semi-dry electroblot system (BioRad, Germany) according to the standard protocols. Briefly, the membranes were blocked with 1% bovine serum albumin. The blotted proteins were separately reacted with mouse anti-HA (ProSci, USA), anti-NA (AbCam, USA) and anti- M1 (SeroTec, UK) monoclonal antibodies diluted 1:200 in TBST for 2 hours at room temperature. The appropriate secondary antibody, goat anti-mouse IgG conjugated with HRP (Razi Fara Teb, Iran) in 1:2000 dilutions, was used as secondary antibody. The protein bounds were developed by staining the membranes with diaminobenzidine (DeMaria *et al.*).

RESULTS

Construction of the Recombinant Bacmid DNA

Following the transformation of DH10Bac cells using pFastBacHTb_PEDV-S1 donor plasmid, the colonies harboring the recombinant bacmid were selected through blue

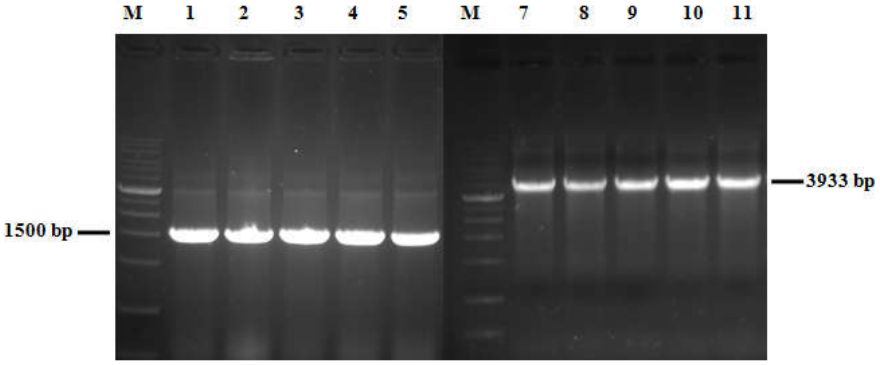

Figure 1. Gel Electrophoresis of PCR Products Carried pFastBacHTb_PEDV-S1 Recombinant Bacmid (Lane 1 to 5: PCR product using PEDV-S1 forward and M13 reverse primers on recombinant bacmid; amplification of a region with 1500 bp in length as expected. Lane 7 to 11: PCR product using forward and reverse M13 primers on recombinant bacmid (3933. Lane M: 14.0 Kb DNA marker (GeneMark)

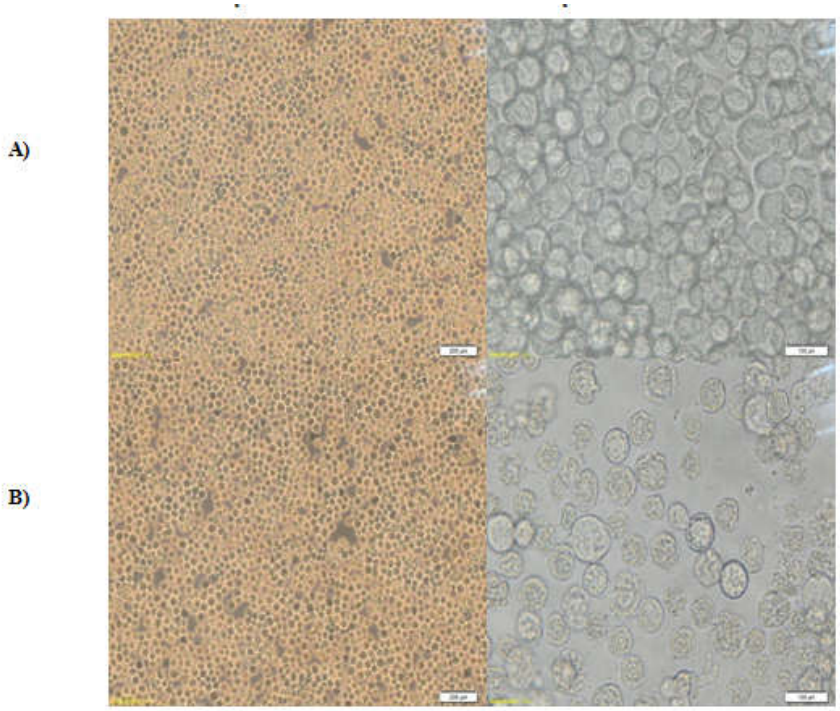

Figure 2. The Mock-Transfected Sf9 Cultures (A, Mock-transfected Sf9 are the cells transfected without DNA to control potential effect of transfection reagent on the cells (negative control). B, Transfected Sf9 cells with recombinant bacmid DNA showing typical cytopathic effects resulted from recombinant baculovirus assembly and propagation

and white screening. The white selected colonies were streaked again on complete medium of LB agar to certify the true phenotype. Since restriction analysis is difficult to perform with high-molecular weight bacmid DNA, PCR was carried out to verify the presence of "HTb_PEDV-S1 cassette" in the recombinant bacmid. The bacmid shuttle vectors containing M13/ pUC forward and reverse hybridization sites flanking the mini-att Tn7 sites within the LacZ region. With respect to position and length of inserted "HTb_PEDV-S1 cassette" into bacmid, the 3930bp regions was amplify using M13/pUC specific primers and S1-forward primer along with M13/pUC-reverse primer in PCR mixtures, respectively (Figure 1).

Transfection of Sf9 Cells

The isolated recombinant bacmid DNA were transfected to Sf9 cells using Cellfectin (Invitrogen).

harvested after 72 hours. Total protein of the cell lysates was electrophoresed on 4 - 12% polyacrylamide gel SDS-PAGE. As shown in Figure 3 the exact polypeptide bands with predicted molecular weight of approximately 55 kDa representing the PEDV-S1 was detected. Western blot analysis of separated proteins on SDS-PAGE, probed with anti-PEDV-S1monoclonal antibodies proved the specificity of the detected bands and also demonstrated simultaneous and independent expression of the new recombinant PED virus protein (Figure 4). However analysis of the recombinant infected cell medium showed no detectable band.

DISCUSSION

The production of PEDV VLPs as a vaccine candidate in insect expression system offers several advantages over current egg-based methods, particularly in terms of handling, robustness, yield, safety and cost efficiency (Park *et al.,* 2004).

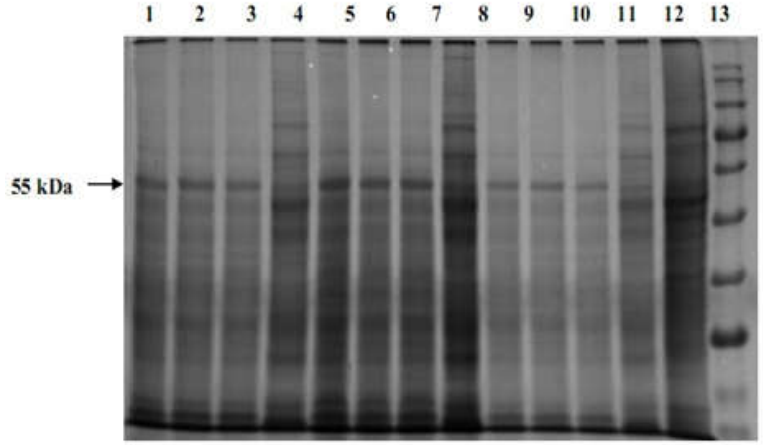

Figure 3. SDS-PAGE Analysis of Protein Extracted From Sf9 Cells Infected by Triple Baculovirus Recombinants

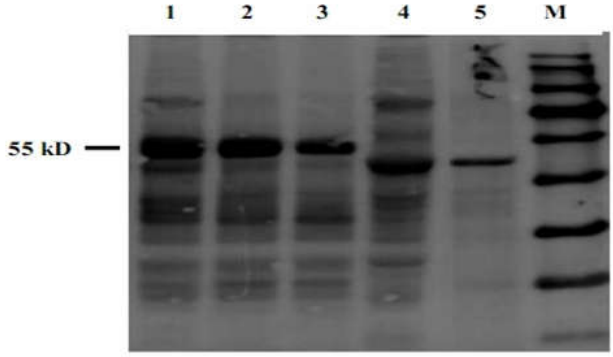

Figure 4. Western Blot Analysis of the PEDV-S1 Protein Expressed in Sf9 Cells Infected by Triple Baculovirus Recombinant

The transfected cells displayed typical cytopathic effects, i.e. low cell density, division stop, enlarged cells and poor adherence to the substrate, indicating that the virus production was taking place. The mock-transfected Sf9 cultures continued to divide and form a confluent normal cell monolayer (Figure 2).

Determination of Recombinant Proteins

To analyze the expression of a recombinant protein, the Sf9 cells were infected with recombinant triple baculovirus and

In this study, we designed and constructed the polycistronic baculovirus expressing envelope proteins of PED virus which is an essential need for PEDV VLP production in insect cell lines. Since the PEDV apparently caused zoonotic infections and currently is endemic in vast areas of the world, we used S1 genomic sequences of this subtype to construct specific recombinant baculovirus that is required for PEDV-S1 VLP production in insect cell system. The baculovirus expression vector system (BEVS) has several features such as it ease to design and construct new gene combinations, the scalability offered by the system, and the possibility of rapid a VLP

vaccine production in an emerging situation (Hitchman *et al.,* 2011; Lopez-Vidal *et al.*, 2015). Several groups have reported the production of PEDV VLPs using baculovirus expression vector in the insect cell system (Hitchman *et al.,* 2009). They used one protein gene to make immunogenic VLPs specific to different PEDV subtype (Hou *et al.,* 2007).

In addition different expression strategies have been used to produce proteins complexes in Baculoviral expression system (BVES) (Hitchman *et al.,* 2009). In some researches, the proteins are expressed from infection of multiple monocistronic baculoviruses (Gotoh *et al.,* 2008) while the others have exploited the use of a single polycistronic baculovirus to generate self-assembling multi-protein complexes expression (Guijarro-Pardo *et al.,* 2014). (Gomez-Sebastian *et al.,* 2014) found that only the expression of tricistronic baculoviruses led to the production of VLPs, and their results were termed "inconclusive" from co- infection experiments. (Felberbaum, 2015) decided to use polycristronic baculoviruses in their VLP studies, too. Additionally, many have used polycristronic baculoviruses as a method of overcoming limitations inherent to co-infection, specifically the uneven distribution of virus taken up by cells. In other word, all essential proteins necessary for the generation of the recombinant product are expressed in the same infected cell by using polycistronic baculoviruses (Hitchman *et al.,* 2011).

According to the mentioned findings and points of view, in this study we designed a compound cassette comprising of three transcriptional unites to generate a recombinant tricistronic baculovirus. Polh and p10 promoters both are used conventionally to generate large quantities of proteins in BVES. The effective performance of polh promoter to drive the expression of two or more genes of PED virus in a single baculovirus has been previously shown (Alonso *et al.,* 2014). On the other hand, to avoid "competition effect" which may occurs when two genes expressed at the same time and at high level, under the control of two different promoters, in the pFastBacHTb_PEDV-S1 cassette, polh promoter has been choose for all transcription unites. SV40 poly-A signal element is widely used in comerial vectors to drive transcriptional termination and improves translational efficiency of inserted genes. In the pFastBacHTb_PEDV-S1cassette, we also used SV40 poly-A signal sequence at the end of each HTb transcription unites but the S1, exploited the SV40 polyadenylation signal belongs to pFastBacHTb that following the insertion of cassette has located immediately downstream of S1. The protein molecular weight of PEDV_S were 120-180 kDa. Nevertheless, the HTb band in the blot appears slightly around 55 kDa which leads to the conclusion that the HTb are glycosylated. Insect cells perform complex glycosylation but in a slightly different manner than mammalian cells. Nevertheless, any negative effect resulted from different pattern of glycosylation on the immunogenicity of the recombinant HTb has not yet been reported in the in vivo studies.

Conclusion

The recombinant baculovirus constructed in this study has proper characteristics that can generate PEDV-S1 VLP in Sf9 cells for vaccine purpose. Experiments are in progress to produce large quantity of purified PEDV-S1 complex proteins to prove formation of VLP in electromicroscopy and assay its immunogenicity in mouse model.

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